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Introduction

Prostate cancer is the most common malignancy in men and is the second leading cause of male cancer related deaths. The multi-step molecular pathogenesis is not clearly defined. Our laboratory has been examining alterations in the interrelated Cdk4/p16 and pRB pathways. We, and others, have reported that the Cdk4 inhibitor p16 is commonly induced in primary prostate cancer. As p16 should be arresting growth it was a surprising finding that it was induced in prostate cancer epithelium. Another gene that is induced in early prostate cancer development is Cdc37. The 50 kDa product of the mammalian *Cdc37* gene is a co-chaperone protein that preferentially recruits protein kinases and is responsible for their activity (1-3). Cdk4 is a well-characterized Cdc37 client kinase. We hypothesized that Cdc37 induction may play a positive growth role and counter p16 induction in prostate cancer. Mechanistically, Cdc37 may bind to and activate more Cdk4 to participate in active cyclin D:Cdk4 complexes, thus negating inactive p16:Cdk4 complexes.

To test this hypothesis and assess the role of Cdc37 in the progression of prostate cancer, I proposed three Specific Aims or Tasks. These Aims were designed to test the association of p16 and Cdc37 induction in prostate cancer tissues and, more directly, to assess the role each of these genes could play in normal and cancer prostate cells in vitro. I have outlined the ongoing experiments below point by point, noting progress and problems. Overall, the project has been very successful and has lead to both technological advancements and significant scientific findings. The study has culminated in a peer reviewed research paper in the journal *Cancer Research* and has served as a basis for an NIH grant submission on June 1, 2003.

Body

Task 1. *To examine Cdc37 levels in focal regions of primary and metastatic prostate tumors and determine if they correlate with p16 expression.*

Rationale: Both p16 and Cdc37 levels are elevated in primary prostate cancer (4-7). If Cdc37, through association with Cdk4, is preventing high levels of p16 from binding Cdk4, then Cdc37 should be upregulated in association with p16. Through analysis of serial sections, individual glands that contain high levels p16 should co-express Cdc37. This aim will determine whether or not Cdc37 levels are able to override the effects of p16 *in vivo* and examine the prognostic value of the Cdc37:p16 relationship in early-stage primary prostate cancer.

Previous goals:

- a. *Obtain antibodies to Cdc37 and optimize immunohistochemistry protocols to allow for specific detection of Cdc37 (this has already been accomplished for p16).*
- b. *Immunohistochemically stain serial sections of 50 primary prostate tumor and normal pairs of tissue for Cdc37 and p16.*

Progress: Two commercially available antibodies were obtained for Cdc37 detection from Santa Cruz Biotechnology (Santa Cruz, CA). Through the University of Wisconsin IHC lab these antibodies were tried under various conditions and tissue types. With the polyclonal antibody (sc-5617) specific nuclear staining could be detected in a subset of cancer specimens (Figure 1). However, in the majority of samples significant background staining was problematic. We contacted Dr. J. Wade Harper at Baylor University to see if we could use the antibody reported in their original manuscript, however they sold the antibody to a company that

subsequently discontinued the item. Future studies warrant the use of an antibody that is of sufficient quality for immunohistochemical techniques.

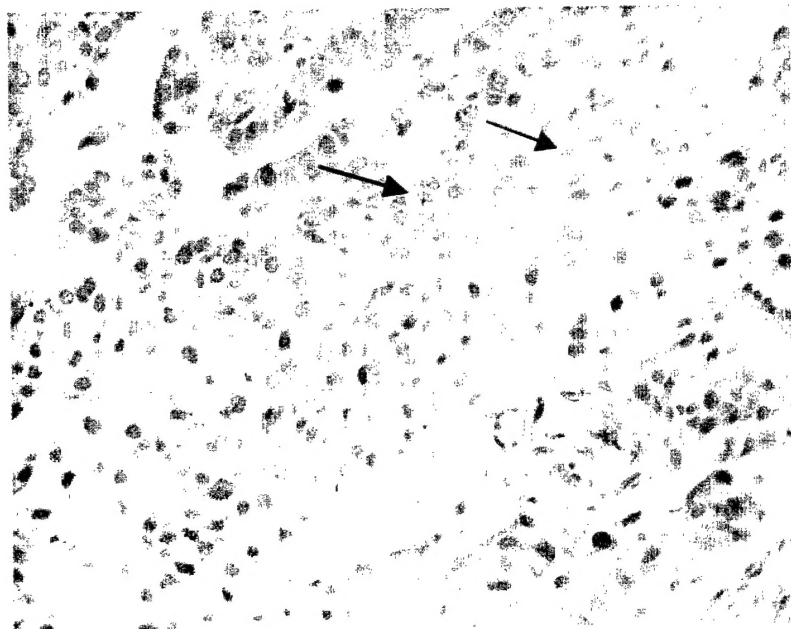


Figure 1. Cdc37 immunostaining. A gleason 10 localized prostate cancer specimen shows intense nuclear and cytoplasmic Cdc37 localization. Note the heterogenous makeup of the tumor with some cells positive (black arrow) while some remain negative (blue arrow).

As an alternative p16 and Cdc37 mRNA levels were assessed in laser capture microdissected tissues. Three patient matched tumor/normal tissue pairs were selected. Cryosections were cut, H&E stained, and Gleason score determined by a urologic pathologist. Only matched normal and tumor specimens were used that contained no or all cancer, respectively. Furthermore, only those specimens with moderately differentiated cancer were selected (Gleason score 5-7). To separate epithelium from stromal components, laser capture microscopy was used. Specimens were cryosections (10 μ m), dehydrated through an ethanol series, and allowed to dry for 3 hours. Epithelium and stromal components were captured using the PixCell II (Acturus, Mountain View, CA) laser capture system. Total RNA was isolated with the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). RNA was precipitated using ethanol and glycogen as a carrier. Each sample was converted into first strand cDNA with SuperScript reverse-transcriptase (Invitrogen, Carlsbad, CA). Real time Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye as described using a iCycler detection system (Bio-Rad)(8, 9). For comparison of transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established. This value was then used to calculate the relative abundance of each gene. These values were then normalized to the relative amounts of 18S cDNA. Quantitative real-time RT-PCR (qRT-PCR) was performed on cDNA generated from each sample. After standardizing to 18S rRNA levels, tumor epithelium consistently expressed more *Cdc37* message than normal epithelium (2-9 fold; Table 1)

Table 1. *Cdc37* mRNA is upregulated in primary prostate cancer. Laser capture microscopy was performed on 3 tumor/normal pairs of tissue obtained from radical prostatectomy. Epithelium was captured and the amount of *Cdc37* and *p16* message was measured using real-time qRT-PCR.

Sample	Gleason score	<i>Cdc37</i> tumor:normalT:N (fold increase)	<i>p16</i> tumor:normalT:N (fold increase)
0110	3+4=7	9.0 \pm 0.7	12 \pm 3.0
0112	3+4=7	2.3 \pm 0.3	5.0 \pm 1.8
0117	3+3=6	3.0 \pm 0.5	32 \pm 3.2

Task 2. To determine if *Cdc37* is able to override *p16* mediated growth arrest.

Rationale: Our laboratory has identified that several prostate carcinoma cell lines are *p16* negative due to promoter methylation (PPC-1, PC-3 and TSU-PR1) (10). *p16* re-expression in these cell lines leads to G₁ arrest and acquisition of a senescent-like state (11). This model provides an excellent system to test this aim since bypassing one factor (*p16*) will allow the cells to continue to proliferate. In addition, the relative levels of *Cdc37* and *p16* can be controlled. The experiment will be carried out by determining a ratio of *Cdk4*:*p16* that just restricts growth. Next, either *Cdc37* or an empty expression vector will be introduced and the number of cells in S-phase determined. If *Cdc37* overexpression is able to raise the number of cells in S-phase over the vector only, then we will conclude that *Cdc37* is able to bypass *p16*-mediated growth arrest.

Previous goals:

- Develop a transfection plasmid for overexpressing *Cdc37* (has already been accomplished for *p16*).
- Transfect *Cdc37* and *p16* plasmids into prostate cancer cell lines and determine the cell cycle status and proliferation levels.

Progress: Human *Cdc37* cDNA was obtained (J. Wade Harper, Baylor College of Medicine). The open reading frame was then cloned into an expression vector (pcDNA3.1). Human *Cdk4* cDNA from our laboratory was cloned into the pBABE puro expression vector. The *Cdk* inhibitor, *p16*, in the GFP expression vector, pLSG, was as described (12). The prostate cancer cell line PPC-1 was transfected using Effectene (Qiagen) with various combinations and ratios of these plasmids to determine if *Cdc37* could override the growth arrest induced by *p16*. Cell cycle analysis of cells was accomplished by analyzing GFP positive cells pre-incubated with the fluorescent DNA dye Hoechst 33342 (Molecular Probes, Eugene, OR). Cell cycle position of GFP (transfected) cells was determined using MODFIT software (Becton Dickinson, San Jose, CA). Expression of all constructs was determined by western blotting.

The results of this experiment are demonstrated in Table 2. It was determined that a *Cdk4*:*p16* ratio of 2:1 was still sufficient to arrest growth, while a ratio of 3:1 began to allow more cells in S-phase. At a *Cdk4*:*p16* ratio of 2:1, *Cdc37* or the empty vector (pBABE puro) were co-transfected. *Cdc37*, however, was not able to push more cells into S-phase. We therefore conclude that in the PPC-1 cell line *Cdc37* cannot overcome *p16* mediated cell cycle arrest.

Sample	DNA transfected					% in S-phase
	GFP	p16-GFP	Cdk4	Cdc37	pCDNA3.1	
GFP only	X					26.9%
Cdc37 only				X		25.9%
p16 only		X				1.6%
Cdk4 only	X		X			27.1%
Cdk4:p16 (1:2)		X	X			5.5%
Cdk4:p16 (1:1)		X	X			5.2%
Cdk4:p16 (2:1)		X	X			5.6%
Cdk4:p16 (3:1)		X	X			11.7%
Cdk4:p16 (2:1) +pCDNA3.1		X	X		X	5.4%
Cdk4:p16 (2:1) +Cdc37		X	X	X		5.9%

Table 2. Cdc37 cannot overcome p16-mediated cell cycle arrest in PPC-1 cells. PPC-1 cells were transfected with the indicated plasmids. At 72 hours post-transfection, cells were labeled with Hoechst 33342 and the DNA content of GFP positive (transfected) cells analyzed by FACS. Clearly, p16 was able to induced cell cycle arrest. Cdk4 was co-transfected to act as a reservoir to bind excess p16 and to form a potential large pool of immature Cdk4 for Cdc37 to fold. A ratio of 3:1 Cdk4:p16 was sufficient to partially alleviate the p16 growth arrest. Therefore a slightly lower Cdk4:p16 ratio of 2:1 was used to test the ability of Cdc37 to bypass the p16-mediated growth arrest. Either Cdc37 or the control pCDNA3 vector was co-transfected with Cdk4 and p16. However, Cdc37 was unable to enhance proliferation.

Task 3. To determine, using an *in vitro* approach, if Cdc37 overexpression is able to extend replicative lifespan in normal human prostate epithelial cells.

Rationale: Studies demonstrate that Cdc37 can be a positive growth regulator *in vivo*, causing prostate epithelium hyperplasia, and can act as an oncogene when combined with cyclin D1 or c-myc overexpression in mice (4, 13). As normal cells progress from proliferation to senescence they accumulate high levels of p16 (12). A necessary alteration for lifespan extension is bypassing p16 by inactivating pRb, mutating or deleting p16, or through gene silencing by p16 promoter methylation (14, 15). This aim will determine if Cdc37 exerts the observed hyperplastic effects and if Cdc37 can promote immortalization in normal HPECs.

Previous goals:

- Develop a retroviral vector for expressing Cdc37.
- Obtain prostate explants from patients and culture normal human prostate epithelial cells.
- Infect prostate epithelial cells with Cdc37 retrovirus and select for infected cells.
- Compare cells infected with Cdc37 and vector only virus. Perform BrdU incorporation assays to address proliferative.

Progress: The bulk of the significant finding thus far came from this Task. There has been a major accomplishment in the development of retroviral production methods, as well as, in the demonstration that Cdc37 can induce proliferation. Further molecular analyses derived from this finding have yielded a wealth of

information regarding the mechanism of Cdc37 induced proliferation in HPECs. A more detailed analysis of this study can be found as a submitted manuscript in the appendix.

(a) Develop a retroviral vector for expressing Cdc37. Currently, transfection of HPECs is very inefficient (less than 0.5%) and therefore this method is not a viable method for gene delivery. Retroviral gene expression is, however, a method that allows stable incorporation of exogenous gene expression in normal cells. Standard methods for retrovirus generation rely on transfecting amphotropic cell lines with a linearized expression construct, followed by drug selection for stable cell lines. A number of stable cell lines are then expanded in culture and the viral titer assayed. Finally, large quantities of viral supernatant are harvested from the highest titer cell line and frozen away. The production of viral supernatant in this manner has several downfalls including:

1. Several months 2-3 are required just to make viral supernatant.
2. Stable cell lines cannot be generated from constructs that induce growth arrest or apoptosis.
3. Freeze-thawing viral supernatant reduced viral titer by 2-fold.
4. Many expressing cell lines have their titer reduced significantly if they are froze down and, therefore, the whole 2-3 month procedure needs to be performed if more supernatant is needed in the future.

As an alternative, Dr. Gary Nolan at Stanford University has devised a vastly improved method for retroviral production. This protocol has been adapted for use in HPECs. Briefly, to generate virus, the DNA vectors were transfected (Effectene, Qiagen) into Phoenix Ampho packaging cell lines (provided by Gary Nolan, Stanford University through ATCC, Manassas, VA) according to the Nolan lab protocol. At day 2 post-transfection, the supernatant was harvested, filtered, 4 μ g/ml polybrene added and placed onto HPECs. After 8 hours, the viral containing supernatant was replaced by F12+. At two days post-infection, HPECs infected with the pBABE puro parent vector were treated with 4 μ g/ml puromycin. Drug treatment was carried out for three days, at which point no uninfected cells remained viable. At day 5 post-infection, cells were re-plated to 50-70% confluency. Infection rate for all constructs varied between 5-22%.

This improved retroviral production methodology has allowed vast improvements in:

1. Time: retrovirus is to be produced in 2 days instead of 2-3 months.
2. Retroviral production from all constructs: retrovirus has been made from the negative growth regulator, p16, and from the apoptosis-inducing construct, Cdc37 Δ C, an impossible feat with standard technologies.
3. Reliable high viral titer: With transfection procedures optimized for the Phoenix Ampho cell line high viral titers are reliable made. Also, the timing of viral production can be synchronized to need, thus preventing loss of viral titer through freeze thawing procedures.
4. Convenience: No need to freeze down retroviral producing cell lines.

(b) Obtain prostate explants from patients and culture normal human prostate epithelial cells. With close collaboration with my sponsor, Dr. David Jarrard, the availability of human prostate tissue has been adequate. With our expertise, no problems have been encountered in culturing normal human prostate epithelial cells.

(c) Infect prostate epithelial cells with Cdc37 retrovirus and select for infected cells. As stated in (a), retroviral infections and expression in HPECs has been very good (5-22% infection rates). Further proof of the ability to infect HPECs is demonstrated in each of the figures in the appendix.

(d) Compare cells infected with Cdc37 and vector only virus. Perform BrdU incorporation assays to address proliferative. Cdc37 overexpression was able to drive proliferation compared to vector only infected HPECs

(66-123%). Ensuing molecular analysis was performed on cellular extracts to gain insights as to mechanistic alterations that were potentially leading to cellular proliferation (Appendix Figure 1). Molecular analysis of Cdc37 client pathways indicated enhanced Raf-1 activity, up-regulated Cdk4 and cyclin D2 levels and lower p16 expression with Cdc37 overexpression. These findings suggest increased Raf-1 and/or Cdk4 activity might underlie the proliferative enhancement (Appendix Figure 1).

To address whether loss of Cdc37 function inhibits growth a dominant negative Cdc37 construct, Cdc37 Δ C, was overexpressed in HPECs. This protein retains the ability to bind kinases and homodimerize, however it cannot bind to Hsp90 due to a C-terminal truncation. Thereby, maturation of Cdc37 client polypeptides is blocked (16). At 4 days post-infection, distinct morphological changes were readily apparent in HPECs expressing Cdc37 Δ C. Cells lost their characteristic cuboidal epithelial morphology, instead becoming contracted, and highly light refractile (Appendix Figure 2A). At 5 days post infection, Hoechst 33342 stained cells were observed by fluorescent microscopy to assess nuclear DNA integrity. An abundance of fragmented, or pycnotic, nuclei suggested the cell death was apoptotic (Appendix Figure 2B). At the same time point, Cdc37 Δ C and control pBABE puro infected cultures were assayed for proliferation by BrdU incorporation. Effective growth cessation (~10-fold) was caused by Cdc37 Δ C expression with cells accumulating in G0/1 (Appendix Figure 2C). This data further demonstrates that Cdc37 is critical for proliferation and is necessary for survival.

To further address which of the observed molecular changes could lead to growth enhancement, the Raf-1 induction, Cdk4 and cyclin D1 overexpression were recapitulated. Constitutive Raf-1 activation however resulted in growth arrest (Appendix Figure 3). The mechanism behind this growth arrest is unclear, however overexpressed activated Raf-1 is unable to result in growth activation. Cdk4 was also overexpressed and was unable to generate a proliferative response. Interestingly, cyclin D1 overexpression was sufficient to promote proliferation. These findings are consistent with they hypothesis that Cdc37 mediates growth arrest through inducing Cdk4 activity.

One of the most surprising findings was that blocking Cdc37 activity resulted in apoptosis. This indicates that Cdc37 is necessary for survival. It would also be an important finding that Cdc37 also performs anti-apoptotic functions. Recently, it was demonstrated that Cdc37 is necessary for activity of the anti-apoptotic gene AKT (17). It is interesting to speculate that Cdc37 overexpression observed in human prostate tissue not only promotes proliferation, but also apoptosis resistance.

Key Accomplishments

- mRNA quantitation of genes using laser capture microdissection and quantitative real-time PCR
- Cloning of all desired cDNA constructs into expression vectors with confirmed gene expression
- Generation of a retrovirus using improved methodology for gene expression in normal human prostate epithelial cells (HPECs)
- Efficient infection and gene expression in HPECs

Reportable Outcomes

1. An accepted manuscript: Schwarze, S.R., Fu, V.X., and D.F. Jarrard. 2003. Cdc37 Enhances Proliferation and is Necessary for Normal Human Prostate Epithelial Cell Survival. *Cancer Res.* 63:4614. The manuscript is attached in the appendix.
2. Applied for an NIH K01 award based on these studies. Priority score 171. Funding decision pending.

Conclusions

1. p16 and Cdc37 mRNA are co-induced in the prostate cancer compared to normal tissue.
2. Cdc37 is not able to overcome p16-mediated growth arrest in PPC-1 cells.
3. Cdc37 overexpression drives proliferation in HPECs.
4. Cdc37 overexpression results in molecular changes consistent with induction of Cdk4 activity.
5. The Cdc37 downstream Cdc37 target cyclin D1 is sufficient to drive proliferation alone.
6. Loss of Cdc37 function leads to growth arrest and apoptosis.

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Appendix Cover Sheet

Cdc37 Enhances Proliferation and Is Necessary for Normal Human Prostate Epithelial Cell Survival¹

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ABSTRACT

Cdc37 is a co-chaperone protein that recruits several immature client kinases to Hsp90 for proper folding. Cdc37 up-regulation is a common early event in localized human prostate cancer. Although targeted overexpression in mice leads to prostate epithelial cell hyperplasia, the effect of Cdc37 dysregulation in human prostate cells is unclear. In this study, we examine the role of Cdc37 in the growth regulation of normal prostate epithelial cells using a unique human model system. We demonstrate that Cdc37 overexpression drives proliferation, whereas loss of Cdc37 function arrests growth and leads to apoptosis. With increased Cdc37 expression, molecular analysis of Cdc37 client pathways demonstrates enhanced Raf-1 activity, greater Cdk4 levels, and reduced expression of the cyclin-dependent kinase inhibitor p16/CDKN2. To further investigate these downstream pathways, enhanced Raf-1 or Cdk4 activities were selectively induced in human prostate epithelial cells. Raf-1 activation inhibited proliferation and generated an enlarged, flattened morphology. Induction of Cdk4 activity using cyclin D1 overexpression, however, was sufficient to promote proliferation. These data indicate that Cdc37 induces proliferation and is critical for survival in human prostate epithelial cells. These alterations in cell division and survival may be important in the development and progression of early prostate cancer.

INTRODUCTION

Cdc37 is a co-chaperone protein that targets and activates multiple protein kinases. These interactions are important for a number of mitogenic signaling pathways. As a critical component of cell cycle control, Cdc37 dysregulation has been implicated in the development of cancer. Recently human prostate tissue specimens were surveyed for Cdc37 expression and increased immunoreactivity was found in all specimens analyzed when compared with normal tissues (1). Cdc37 overexpression was also found in the luminal cells of the prostate cancer precursor lesion, PIN,³ indicating Cdc37 activation may be an important early step in prostate cancer development. To extend these findings, targeted Cdc37 overexpression in mouse prostate epithelium using the probasin promoter generated epithelial hyperplasia in the ventral prostate by 8 months of age (1). In mice Cdc37 has also been found to collaborate with *c-myc* in the development of tumors in multiple tissues suggesting it regulates a rate-limiting step in epithelial cell transformation (2). However, the effect of Cdc37 on the proliferation and survival of primary human epithelial cells, cells in which *in vitro* and *in vivo* transformation is rare, is unknown.

The M_r 50,000 product of the mammalian *cdc37* gene is a co-

chaperone that is absolutely required for Hsp90 substrate-specific folding activity (3–6). Hsp90 family members are molecular chaperones that provide maturation and folding to a number of client polypeptides in an ATP-dependent manner. Cdc37 binds immature protein kinases through interaction with its NH₂-terminal region (7) and links these to the Hsp90 COOH terminus (8). In addition to a physical role in targeting kinases to Hsp90 for activation, yeast CDC37 also exhibits protein chaperone activity (4) opening the possibility that the mammalian homologue may also possess some folding activity. Biologically, Cdc37 is critical not only for kinase activation but also protein stability. Blocking Cdc37 function in immortalized cell lines using either a dominant negative Cdc37 mutant (7) or inhibiting Hsp90 activity with ansamycins, such as geldanamycin (9), results in both decreases in steady-state protein levels and in the activity of client kinases, such as Cdk4, Raf-1, v-Src, Akt, and the androgen receptor (7, 10–13).

One Cdc37 client kinase, Cdk4, is a critical component of the cell cycle machinery. The decision to progress through the cell cycle is modulated by a series of signal transduction pathways acting on genes required for cell cycle progression. Cyclin D proteins (D1, D2, or D3) form an active kinase complex with Cdk4 or Cdk6 that phosphorylates pRB and functions in the G₁ to S phase transition (14). These positive proliferation signals are antagonized by p16 and other members of the cyclin-dependent inhibitor family INK4a, which compete with cyclin D for binding to Cdk4 and Cdk6, thereby preventing kinase activity (15). Overexpression of either cyclin D1 or replacement of wild-type Cdk4 with a Cdk4 mutant that cannot bind the CDK inhibitor p16, Cdk4 R24C, leads to elevated Cdk4 activity, induction of proliferation, and elevated tumor incidence in mice (16, 17). Furthermore, fibroblasts derived from Cdk4 R24C homozygous mice or p16 knockout mice are immortal in culture (17). Thus, regulation of Cdk4 activity is critical in the proliferation of normal cells and appears to play a role in cancer susceptibility at least in mouse models.

Another Cdc37 client kinase is Raf-1, a critical signaling molecule in the mitogen-activated protein kinase pathway that transmits information from the cell surface to the nucleus and cell cycle machinery. Signaling through Raf-1 is commonly initiated by the membrane-bound family of Ras GTPases after stimulation by peptide ligands and growth factors resulting in receptor tyrosine kinase activation (18, 19). In the GTP-bound state, Ras recruits Raf-1 to the membrane, a process that results in Raf-1 activation (20). Activated Raf-1 can then phosphorylate MEK1/2, which can then phosphorylate and activate the p42/p44 mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 (21). Signaling through Raf-1 positively regulates proliferation likely via the stimulation of cyclin D1 expression (22). Paradoxically, overexpression of activated Raf-1 can also lead to growth arrest and differentiation in both primary and immortalized cells (23, 24). It is unclear if these proliferative or inhibitory signals are cell type specific or dependent on the immortalization status.

In the present study we examine the role of Cdc37 in the proliferation of genetically intact nonimmortalized human epithelial cells using a unique cell model (25). Consistent with mouse transgenic studies, Cdc37 overexpression in HPECs is sufficient to enhance proliferation. Furthermore, Cdc37 inhibition results in an apoptotic

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³ The abbreviations used are: PIN, prostatic intraepithelial neoplasia; HPEC, human prostate epithelial cell; BrdU, 5-bromo-2-deoxy-uridine; PBST, PBS plus 0.1% Tween 20; RT-PCR, reverse transcriptase-PCR; CDK, cyclin-dependent kinase; ΔRaf:ER, Raf-1 estrogen receptor fusion construct; MEK, mitogen-activated protein kinase/extracellular signal-regulated protein kinase.

response suggesting a role for Cdc37 in cell survival. This study supports the hypothesis that Cdc37 overexpression in the prostate can positively regulate growth, inhibit cell death, and play an early role in the progression of human prostate cancer.

MATERIALS AND METHODS

Primary Cell Culture. Prostate tissue was obtained under an approved Institutional Review Board protocol from men (ages 44–66) undergoing cystoprostatectomy for bladder cancer at the University of Wisconsin Hospital and Clinics. Histology confirmed that no bladder or prostate cancer was present in the prostate tissue harvested for our studies. Prostate epithelial cultures were established as described previously (26). Prostate tissues were minced with a scalpel and digested in a solution containing collagenase (500 units/ml; Sigma, St. Louis, MO) and plated on collagen-coated plates. Cells were maintained in Ham's F-12 media (Invitrogen, Carlsbad, CA) supplemented with 0.25 units/ml regular insulin, 1 μ g/ml hydrocortisone, 5 μ g/ml human transferrin, 2.7 mg/ml dextrose, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10 ng/ml cholera toxin, 25 μ g/ml bovine pituitary extract, and 1% FBS (27). Cells were passaged using trypsin-EDTA.

Retroviral Infection of HPECs. To generate virus, the DNA vectors were transfected using Effectene (Qiagen, Valencia, CA) into Phoenix Ampho packaging cell lines (provided by Gary Nolan, Stanford University through American Type Culture Collection, Manassas, VA) according to the Nolan lab protocol.⁴ At day 2 post-transfection, the supernatant was harvested, filtered, added to with 4 μ g/ml Polybrene (Sigma), and placed onto HPECs. After 8 h the viral-containing supernatant was replaced by F-12+. At 2 days postinfection HPECs infected with the pBABE puro parent vector were treated with 4 μ g/ml puromycin (Invitrogen). Cells infected with the Δ Raf:ER vector were treated with 300 μ g/ml Genetically (Invitrogen) with or without 1 μ M β -estradiol (Sigma). Drug treatment was carried out for 3 days, at which point, no uninfected cells remained viable. At day-5 postinfection cells were replated to 50–70% confluency. Infection rates for all constructs routinely varied between 5 and 22%.

Generation of cDNA Clones and Retroviral Stocks. Human *cdc37* cDNA (provided by J. Wade Harper, Baylor College of Medicine) was cloned into the retroviral expression vector pBABE puro. The Cdc37 Δ C dominant negative construct was designed to encompass AA1–164 as described previously (7). The NH₂-terminal fragment was PCR amplified using Expand proofreading DNA polymerase (Roche Biochemicals, Indianapolis, IN), cloned into pBABE puro, and sequenced. The Δ Raf:ER vector (generated by Martin McMahon) consists of the human *Raf-1* gene lacking the NH₂-terminal regulatory domain fused to the human estrogen receptor hormone-binding region. The construct has neomycin as a resistance marker and has been described previously (28). Human Cdk4 and cyclin D1 were PCR amplified from HPEC DNA with Expand proofreading DNA polymerase, cloned into pBABE puro, and sequenced.

SA- β -Gal Staining. Cells growing in collagen-coated p35 dishes were washed twice in 1 \times PBS and fixed in a PBS-buffered solution of 2% paraformaldehyde/0.2% paraformaldehyde for 5 min. Cells were washed again in 1 \times PBS and stained 16 h at 37°C in a solution as described (29).

BrdU Labeling and Cell Cycle Analysis. Cells were fed with fresh media 24 h before BrdU labeling. HPECs were BrdU labeled (Sigma) for 1 h, harvested, and processed using an anti-BrdU monoclonal primary antibody followed by a goat antimouse FITC-conjugated secondary according to the manufacturer's directions (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Cells were analyzed with a FACScan (Becton-Dickinson Immunocytometry Systems) and the percentage of BrdU-positive cells (10,000 gated events) was determined using CellQuest software (Becton-Dickinson Immunocytometry Systems). Cell cycle phase was determined using MODFIT software (Becton-Dickinson Immunocytometry Systems).

Western Blot Analysis. Western blots were performed as described (27) from three independent infections. Cells were harvested by trypsin-EDTA and washed in 1 \times PBS. Protein was extracted by freeze thawing three times in ECB buffer [50 mM Tris (pH 8.0), 125 mM NaCl, 100 mM NaF, 0.5% NP40,

200 μ M Na₃VO₄, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 50 μ g/ml phenylmethylsulfonyl fluoride]. Protein extracts were quantified using the Bradford assay. Twenty-five μ g of whole cell extract were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked in 5% nonfat dry milk in PBST. Polyclonal antibodies to Cdk4 (C-22), Cdk6 (C-21), and Raf-1 (C-20), in addition to monoclonal antibodies to cyclin D1 (A-12) and Cdc37 (N-18), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody to p16^{INK4A} (AB-1) was obtained from Calbiochem (La Jolla, CA). The monoclonal antibody to α -tubulin (AB-1) was obtained from Oncogene (Cambridge, MA). Monoclonal antibodies to phospho S217/221 MEK1/2 and MEK1/2 were obtained from Cell Signaling Technologies (Beverly, MA). Secondary goat antimouse IgG (horseradish peroxidase conjugate) and goat antirabbit IgG (horseradish peroxidase conjugate) antibodies were obtained from Pierce (Rockford, IL). Antibodies were applied in 2.5% nonfat dry milk in PBST for 1 h and washed three times for 10 min in PBST. As a loading control, α -tubulin immunoblotting was carried out on each filter. Bound antibody was detected using chemiluminescence (Pierce).

RT-PCR. Total RNA was isolated from HPECs infected with either Cdc37 Δ C or the pBABE puro backbone virus 5 days postinfection with the RNeasy total RNA isolation kit (Qiagen). First-strand cDNA was generated with SuperScript reverse transcriptase (Invitrogen) from 1 μ g of total RNA. The primer sequences used to amplify a segment of the Cdc37 NH₂ terminus were 5'-AAGGAAAGATGGTGGACTACAGC-3' and 5'-TCACATGCAAGTGCTTGA-3'. After 30 PCR cycles, reactions were electrophoresed on an agarose gel, stained with Sybr Green (Molecular Probes), and visualized with the Storm imaging system (Molecular Dynamics, Piscataway, NJ).

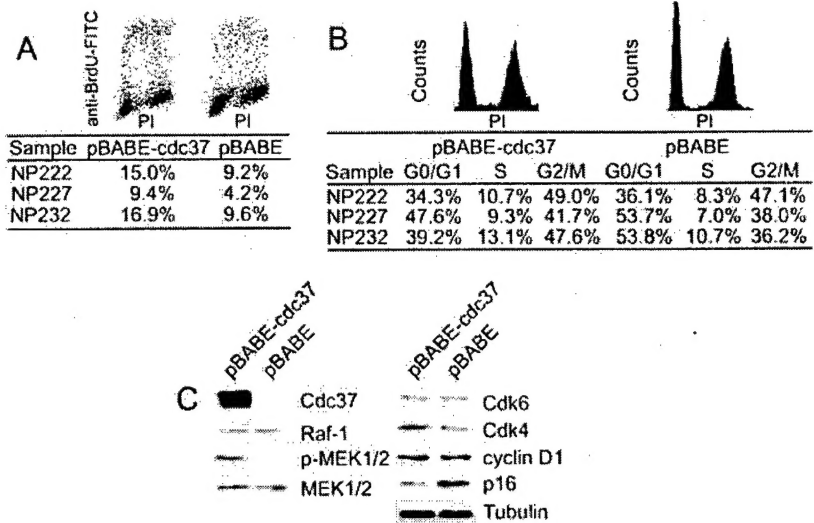
RESULTS

Cdc37 Overexpression Increases BrdU Incorporation in HPECs. Using a retroviral system, Cdc37 or the empty pBABE puro vector was expressed in primary HPEC cultures. Infected cells were selected with puromycin until no viable cells remained in the uninfected cultures (72 h). No morphological changes were apparent between Cdc37 overexpressing and control cells (data not shown). To determine the percentage of cells actively undergoing DNA synthesis, cells were labeled with the nucleotide analogue BrdU, a commonly used method to quantitate proliferation. At 8 days postinfection, HPECs were pulsed with BrdU for 1 h, labeled with an anti-BrdU antibody, and analyzed by fluorescence-activated cell sorting (Fig. 1). Cells overexpressing Cdc37 showed a consistent marked increase in the number of BrdU-positive cells compared with the pBABE vector-infected control cells (66–123% increase; Fig. 1A) and a decrease in the number of cells in G₀-G₁ (Fig. 1B).

Cdc37 Overexpression Leads to Alterations in Raf-1 Activity and Modulates the Cdk4 Pathway. We next surveyed for molecular changes that may lead to enhanced proliferation in Cdc37-overexpressing HPECs. Because Raf-1 and Cdk4 are known Cdc37 client kinases, we focused on genes involved in these pathways. Western blot analysis was performed on protein extracts isolated from HPECs overexpressing Cdc37 or the vector only (Fig. 1C). Cdc37 protein was overexpressed at high levels compared with endogenous levels. Raf-1 steady-state levels were unchanged. However, Raf-1 activity, measured by the abundance of phosphorylated S217/221 MEK1/2, a direct target of Raf-1 (30), was consistently elevated in Cdc37-overexpressing cells, whereas total MEK1/2 levels were unchanged. Increases in Cdk4 levels and markedly reduced p16 expression were also consistently observed in Cdc37-overexpressing HPECs. No changes in cyclin D1 or Cdk6 expression were detected. Cyclin D2 and D3 were not detectable in HPECs at the protein level. Thus, Cdc37-driven proliferation may be mediated through increased Raf-1 and/or Cdk4 activity.

⁴ Internet address: http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html.

Fig. 1. Cdc37 overexpression increases proliferation and alters expression of cell cycle-related genes. In A, primary HPECs from individual patients were retrovirally infected with a Cdc37 construct or vector only control, selected with puromycin, and analyzed for proliferation through monitoring BrdU incorporation at 8 days postinfection. The values reflect the percentage of cells staining positively with an anti-BrdU antibody indicating DNA synthesis. In B, cell cycle analysis of the infected HPEC cultures was modeled using MODFIT software. This analysis demonstrates a consistent decrease in the G_0 - G_1 DNA content in cells overexpressing Cdc37 compared with the pBABE puro vector only cells. In C, cellular extracts were immunoblotted for known Cdc37 targets and genes involved in the Cdk4/6 pathway. Greater p-MEK1/2 and Cdk4 levels were consistently found in Cdc37-overexpressing protein extracts, as well as decreases in p16 expression. The p-MEK1/2 antibody detects phosphorylated S217 and S221 residues.



Blocking Cdc37 Function Leads to Growth Arrest and Apoptosis. To address whether loss of Cdc37 function inhibits proliferation in normal epithelial cells a dominant negative Cdc37 construct, Cdc37 Δ C, was used. This protein retains the ability to bind protein kinases and homodimerize. However, it cannot associate with Hsp90 because of a COOH-terminal truncation; leading to a block in the maturation of Cdc37 client polypeptides (7). By 4 days postinfection, distinct morphological changes were readily apparent in HPECs expressing Cdc37 Δ C when compared with control cells. The majority of mutant Cdc37-expressing cells lost their characteristic cuboidal epithelial morphology and became contracted and highly light refractile (Fig. 2A). Hoechst 33342-stained cells were analyzed by fluorescent microscopy to assess nuclear DNA integrity. An abundance of fragmented, or pyknotic, nuclei indicated the mechanism of cell death was apoptotic (Fig. 2B). By 8 days postinfection, only 10–25% of Cdc37 Δ C-expressing cells remained attached (data not shown).

Cdc37 Δ C and control pBABE puro-infected cultures were assayed for proliferation by BrdU incorporation at 5 days postinfection. Effective growth cessation (~10-fold) was caused by Cdc37 Δ C expression, with cells accumulating in G_0 - G_1 (Fig. 2C). Existing antibodies

recognizing epitopes in the COOH terminus were unable to detect the COOH-terminally truncated Cdc37. Therefore, RT-PCR was used to amplify a segment of the NH₂ terminus to confirm Cdc37 Δ C expression (Fig. 2D). Western analysis demonstrated that levels of the Cdc37 client kinases Raf-1 and Cdk4 were consistently reduced when compared with empty vector indicating the Cdc37 Δ C-truncated protein was indeed preventing wild-type Cdc37 activity (Fig. 2D). In addition, endogenous Cdc37 expression was elevated in Cdc37 Δ C-expressing cells, suggesting post-translational mechanisms can regulate Cdc37 expression (Fig. 2D). Thus, the Cdc37 dominant negative construct further demonstrates Cdc37 is critical for proliferation in HPECs and additionally plays a role in cell survival by preventing apoptosis.

Raf-1 Activation Causes Growth Arrest. Selective induction of specific kinase pathways activated by Cdc37 was then performed. To test if overexpressing Raf-1 alone could generate a proliferative response, an inducible activated Δ Raf:ER was stably incorporated into HPECs through retroviral infection. After infection, cells were drug selected and Raf-1 activity was induced with β -estradiol. After 6 days of Raf-1 induction cells acquired an enlarged, flattened morphology

Fig. 2. Blocking Cdc37 function inhibits proliferation and leads to apoptotic death. In A, the Cdc37 dominant negative construct, Cdc37 Δ C or a vector only control, pBABE puro, was retrovirally expressed in HPECs. Phase-contrast microscopy demonstrates that Cdc37 Δ C-infected cells lose their cuboidal morphology characteristic of HPECs in contrast to cells infected with control virus at 4 days postinfection. Magnification is $\times 100$. In B, HPECs expressing Cdc37 Δ C or pBABE puro were harvested, fixed, and stained with Hoechst 33342 and analyzed under fluorescent microscopy. Fragmented nuclei, indicative of apoptosis, were abundant in the Cdc37 Δ C-expressing cells and rare in vector only cells. The percentages of fragmented nuclei are shown \pm SD from three separate infections. In C, BrdU analysis and propidium iodide staining demonstrate a marked proliferation reduction and an increase in the number of cells in G_0 - G_1 in HPECs infected with Cdc37 Δ C compared with pBABE puro only controls at 5 days postinfection. In D, RT-PCR directed at the NH₂ terminus was used to demonstrate that the Cdc37 Δ C was expressed (*N-Cdc37*). Antibodies do not react with the COOH-terminal truncated Cdc37 protein. Immunoblotting for the known Cdc37 target genes *Raf-1* and *Cdk4* indicates that the Cdc37 Δ C is functional as the steady-state levels of these target genes are reduced. Endogenous, wild-type Cdc37 levels (*wt-Cdc37*), conversely, were elevated by Cdc37 Δ C overexpression. No alteration in the level of p16 was detected.

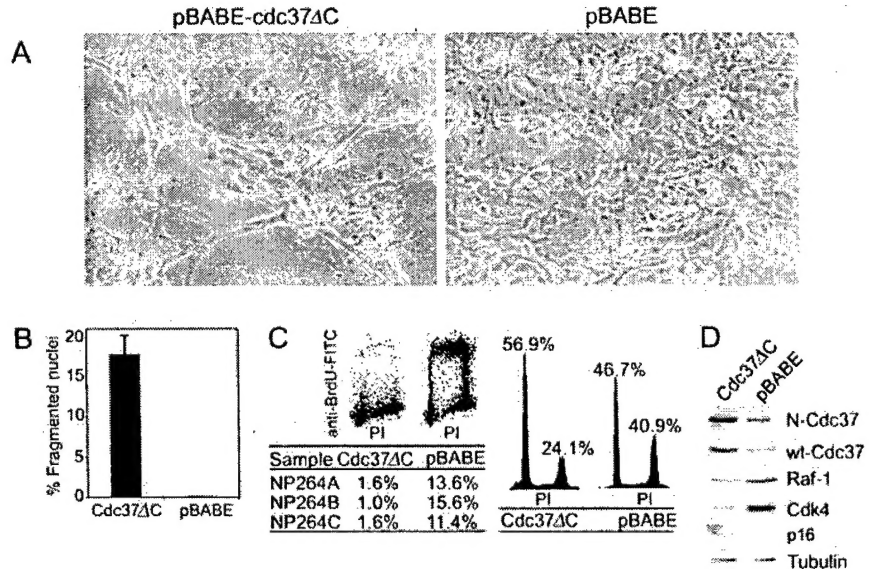
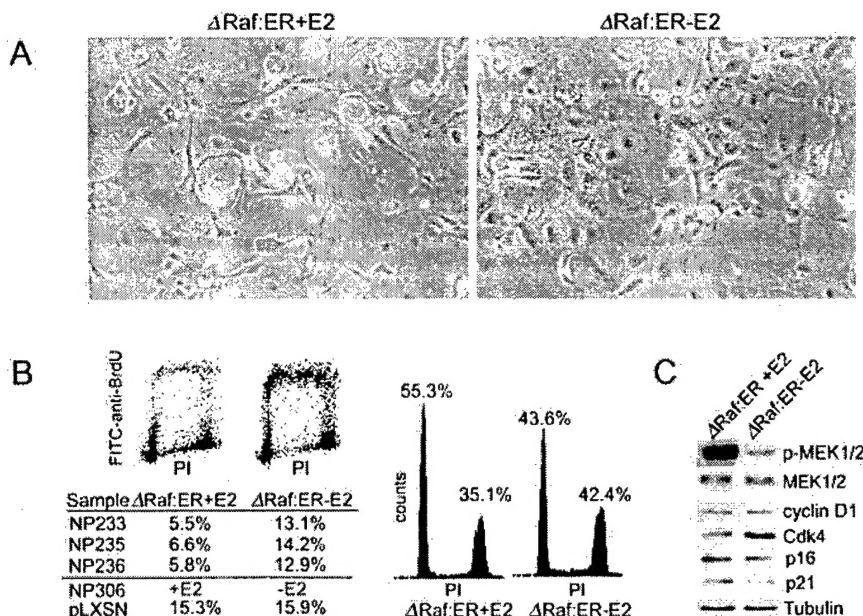


Fig. 3. Activated Raf-1 inhibits proliferation and leads to differentiation. HPECs were retrovirally infected with the β -estradiol inducible Δ Raf:ER construct and selected with puromycin. In A, at 6 days postinduction, cells exhibited noticeable morphological alterations, becoming elongated with the presence of large vacuoles in many cells. In B, BrdU labeling revealed β -estradiol-induced growth arrest with a tendency toward a G_0 - G_1 block in Δ Raf:ER-infected cells. The presence of β -estradiol on vector only infected cells (NP306 pLXSN) did not have an effect on cell growth. In C, molecular analysis shows S217/S221 MEK1/2, the downstream target of Raf-1, is hyperphosphorylated. Investigation into genes involved in modulating Cdk4 activity did not show a difference in cyclin D1, p16, or p21 levels, although Cdk4 expression was modestly decreased with activated Raf-1 overexpression.



(Fig. 3A). BrdU labeling demonstrated decreased proliferation and an accumulation of cells in G_0 - G_1 with Raf-1 activation (Fig. 3B). Western blot analysis showed that the Raf-1 target, S217/221 MEK1/2, is highly phosphorylated; indicating Raf-1 induction compared with vehicle only Δ Raf:ER-infected cells (Fig. 3C). Further analysis of genes involved in G_1 -S transition shows Cdk4 expression to be repressed with Δ Raf:ER induction, whereas p16, p21, and cyclin D1 protein expression remained unchanged.

The growth arrest and morphological alterations observed with Raf-1 induction are characteristic of the phenotype associated with cellular senescence (26, 29). We did not, however, observe positive staining for senescence-associated β -galactosidase (data not shown), a marker frequently but not exclusively associated with this phenotype (29, 31). Thus, activated Raf-1 overexpression leads to the onset of growth arrest, a flattened, elongated morphology, and a nonapoptotic response.

Cdk4 Is Insufficient to Foster Proliferation. However, Cyclin D1 Overexpression Alone Is Sufficient to Generate a Proliferative Response. Molecular analysis of Cdc37-overexpressing HPECs suggests that the observed proliferative increases involve enhanced Cdk4 activity. Both Cdk4 protein increases and p16 repression may contribute to this increase. We manipulated HPECs by two methods to address the impact greater Cdk4 activity has on proliferation. First, HPECs were infected with Cdk4 or the pBABE puro control vector and selected with puromycin. Cdk4 was unable to reproducibly induce an increase in the BrdU proliferation index (Fig. 4A). However, consistently fewer cells accrued in G_1 and a correspondingly higher G_2 -M DNA content was found (Fig. 4B).

Given the difficulty of abrogating p16 expression in primary cell cultures, we used cyclin D1 as an additional molecular tool to determine whether elevated Cdk4 activity could induce proliferation in HPECs. Increasing levels of the Cdk4/6-binding partner cyclin D1 is an established mechanism for increasing Cdk4 activity (16, 32, 33). After drug selection, infected HPECs were assayed for proliferation by BrdU incorporation. Cyclin D1 generated a marked proliferation induction (51–94% higher; Fig. 5A) leading to both decreases in the G_0 - G_1 and G_2 -M DNA content (Fig. 5B). Cellular extracts confirmed cyclin D1 overexpression in transfected cells (Fig. 5C). These experiments indicate that cyclin D1 generates a proliferative response

similar to Cdc37 and Cdk4 activity is rate limiting in HPEC cell cycle progression.

DISCUSSION

It is generally accepted that cancer arises from a combination of uncontrolled proliferative cues, as well as inhibition of cell death pathways. As normal prostate cells transform into high-grade PIN lesions, a putative prostate cancer precursor, there is an increase in the number of cells proliferating (34). Progression to localized prostate

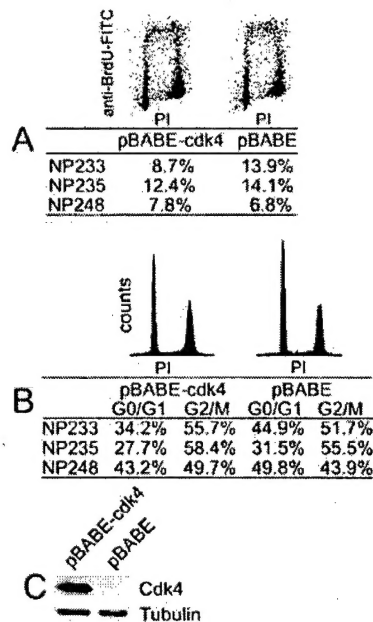


Fig. 4. Cdk4 overexpression is insufficient to induce proliferation. In A, HPECs retrovirally overexpressing Cdk4 or the vector only were assayed at 8 days postinfection by BrdU analysis. Cdk4 overexpression did not result in proliferative increases. B, the cell cycle profile determined by propidium iodide staining. Note consistently fewer cells in G_0 - G_1 in Cdk4-infected cells compared with the patient-matched control pBABE puro-infected cells. In C, immunoblotting of the cellular extracts shows Cdk4 to be overexpressed.

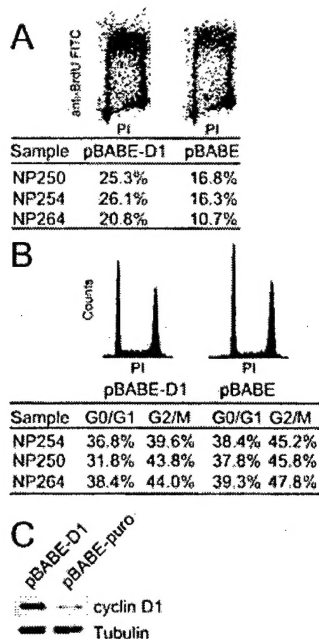


Fig. 5. Cyclin D1 overexpression is sufficient to drive proliferation. In A, HPECs retrovirally overexpressing cyclin D1 or the vector only were assayed at 8 days postinfection by BrdU analysis. In B, the cell cycle profile determined by propidium iodide staining. Note fewer cells in both the G_0 -1 and G_2 phases in cells overexpressing cyclin D1. C, immunoblotting analysis demonstrating cyclin D1 overexpression.

cancer involves no additional increases in proliferation when compared with normal epithelial cells but a decrease in cell death (34). Cdc37 expression is induced in PIN and up-regulated in localized prostate cancer (1). This gene is critical in modulating several cell growth pathways and may have a direct impact on both enhanced proliferation and evasion from apoptosis (7, 10, 12). Our laboratory has a longstanding interest in understanding how genes involved in the G_1 cell cycle checkpoint regulate growth in HPECs in the progression to cancer. In this study we examined the role of Cdc37 in normal human primary prostate epithelial cell growth *in vitro*.

We document for the first time that Cdc37 overexpression leads to a consistent, marked increase in the proliferation rate of normal HPECs. Our findings are consistent with the demonstration that targeted Cdc37 overexpression in the mouse prostate (PB-Cdc37.1 line) leads to the development of epithelial hyperplasia and dysplasia in >50% of prostatic acini (1). The probasin promoter fragment used in the transgenic mouse study is targeted to luminal epithelial cells. However, because secretory luminal cells are terminally differentiated, a proliferating luminal precursor cell may be induced (35). Our collagen-based model represents a proliferative population of epithelial cells that express both basal and luminal markers based on our previous work and others (26, 36, 37). This characterization is consistent with an amplifying or intermediate population of cells *in vivo* that express characteristics of both basal and luminal cells and has been proposed to represent a cell of origin for prostate cancer (38). Thus, Cdc37 induction in HPECs reproduces several aspects of human disease.

A second important observation was that the inhibition of Cdc37 function not only halted proliferation but also efficiently induced apoptosis. This result is not completely surprising as *CDC37*-null *Saccharomyces cerevisiae* are not viable (39). This finding raises the possibility that Cdc37 overexpression may also be antiapoptotic. A role for Cdc37 in the resistance to apoptosis is supported by recent reports demonstrating that Cdc37 binds to, stabilizes, and is required

for maximal Akt activity (12). Because Akt is part of an important survival pathway, reduced Akt activity may sensitize primary HPECs to cell death. It is possible that both higher proliferation rates and apoptosis resistance underlie the hyperplastic growth seen in mouse prostate tissues overexpressing Cdc37 (PB-Cdc37.1) and in human prostate cancers (1). Additional studies will determine whether Cdc37 overexpression can also confer apoptosis resistance *in vitro*.

Our analysis of Cdc37-overexpressing HPECs demonstrated the activation of several growth control pathways, in conjunction with an increase in proliferation, including Raf-1. Raf-1 activity, measured by phosphorylation of its downstream target MEK1/2, was induced in Cdc37-overexpressing cells. Activated Raf-1 overexpression can induce a differential effect in various cell types. In a number of immortal human and rodent cell lines, Raf-1 activation induces malignant progression (28). Conversely, activated Raf-1 uniformly induces growth arrest in finite life span human cells, although arrest also occurs in selected immortalized cell lines, including the prostate cancer cell line LNCaP (23, 24, 40). The mechanism behind Raf-1-induced growth arrest is unclear; however, it does not appear to require intact p53 or pRB pathways (23, 24). This differential response to overexpressed Raf-1 may be derived from genetic alterations that occur during the conversion process from a finite life span to immortalization. Strikingly consistent with previous studies in primary mammary cells, we found activated Raf-1 overexpression resulted in growth inhibition, similar morphological alterations, and a lack of senescence-associated β -galactosidase staining (24). The only cell cycle-related molecular alteration that we observed was a decrease in Cdk4 expression with Raf-1 induction. We do not, however, know what led to this repression or if it contributes to growth arrest. It is important to point out that phosphorylated MEK1/2 levels generated by overexpression of activated Raf-1 are significantly greater than levels derived by Cdc37 overexpression. We cannot rule out the possibility that modest, physiological Raf-1 activity elevations confer greater proliferation rates.

We found that Cdc37 overexpression induces Cdk4 expression and decreases p16 levels, strongly suggesting that an increase in Cdk4 kinase activity occurs. Unfortunately, because of low cell numbers after infection and selection, a limitation of working with mortal HPECs, we were unable to directly assay Cdk4 activity using kinase assays. To model increased Cdk4 activity, we overexpressed the regulatory Cdk4-binding partner, cyclin D1, which results in an increase in proliferation similar to that seen with Cdc37. Increasing levels of the Cdk4/6 binding partner cyclin D1 is an established mechanism for increasing Cdk4 activity (16, 32, 33). Overexpressing activated Cdk4 was not sufficient to confer cellular proliferation in HPECs, indicating that Cdk4 levels are not rate limiting, a finding noted in other primary cells (32). In addition, our finding that epithelial cells overexpressing Cdk4 arrest in G_2 suggests they are able to partially bypass G_1 but require additional factors to pass through G_2 . In sum, these data are consistent with the hypothesis that Cdc37 expression induces Cdk4 activity and that increased Cdk4 activity promotes proliferation in normal HPECs. However, as Cdc37 associates with other known and likely other uncharacterized client kinases, the manner through which Cdc37 promotes HPEC growth is likely multifactorial.

Because the Hsp90/Cdc37 association is important for both proliferation and survival, it is a potential target in cancer therapy (41). The drug 17-allyl-amino, 17-demethoxy-geldanamycin (17-A-GA) indirectly destabilizes kinases that cells need for survival by inhibiting Hsp90 and its related family members (9). Phase I clinical trials for the use of 17-A-GA are currently ongoing (42). However, selectivity between cancer cells and normal cells may be difficult because Hsp90 provides critical functions for a number of normal cellular processes.

A more direct and selective target may be Cdc37. Blocking Cdc37 co-chaperone activity would inhibit growth in overexpressing cells, such as prostate tumors, yet still allow Hsp90 to associate with other biologically important cochaperones, such as p23, HOP, and Hsp70. This Cdc37-specific strategy may result in reduced cytotoxicity compared with anti-Hsp90 drugs. The current findings in our prostate epithelial cell model support a strategy that inhibiting Cdc37 has therapeutic value.

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